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Tsix Transcription- versus RNA-Based Mechanisms in *Xist* Repression and Epigenetic Choice

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Summary

Recent inquiries have revealed a surprisingly large number (>2500) of naturally occurring antisense transcripts [1–4], but their function remains largely undiscovered. A better understanding of antisense mechanisms is clearly needed because of their potentially diverse roles in gene regulation and disease [5–8]. A well-documented case occurs in X inactivation, the mechanism by which X-linked gene expression is equalized between XX females and XY males [9]. The antisense gene *Tsix* [6] determines X chromosome choice and represses the noncoding silencer, *Xist* [10–12]. In principle, *Tsix* action may involve RNA, the act of transcription, or local chromatin. Here, we create novel *Tsix* alleles to distinguish transcription- versus RNA-based mechanisms. When *Tsix* transcription is terminated before *Xist* (*Tsix*^{TRAP}), *Tsix* cannot block *Xist* upregulation, suggesting the importance of overlapping antisense transcription. To separate the act of transcription from RNA, we knocked in *Tsix* cDNA in the reverse orientation (*Tsix*^{cDNA}) to restore RNA levels in *cis* without concurrent transcription across *Xist*. However, *Tsix*^{cDNA} cannot complement *Tsix*^{TRAP}. Surprisingly, both mutations disrupt choice, indicating that this epigenetic step requires transcription. We conclude that the processed antisense RNA does not act alone and that *Tsix* function specifically requires antiparallel transcription through *Xist*. A mechanism of transcription-based feedback regulation is proposed.

Results and Discussion

Potential Mechanisms of *Tsix* Action

Tsix provides a probable paradigm for understanding general antisense action. A priori, antisense genes can exert their influence at any gene expression stage, such as transcription initiation, elongation, RNA processing, RNA stability, and translation. *Tsix* functions in two distinct ways: it chooses the future active (Xa) and inactive (Xi) X [6] and represses Xist RNA, whose "coating" of the X initiates silencing [13, 14]. In female embryonic stem (ES) cells, silencing proceeds on the Xi only when *Tsix* is downregulated, and conversely, activity of the Xa requires persistent expression of *Tsix* in *cis*.

What is the nature of the opposing interplay between *Tsix* and *Xist*? Three classes of mechanisms, though not mutually exclusive, have been proposed (Figure 1A) [15]. In Class I, *Tsix* repression of *Xist* may result primarily from binding of transcription factors to or formation of special chromatin in *Tsix*. "Enhancer competition" based on a regulatable chromatin insulator (CTCF) in *Tsix* has been proposed as a mechanism of establishing choice [16]. Class II mechanisms are based on the observation that transcription also plays a role; forced expression of *Tsix* or its truncation soon after initiation alters its function [13, 14]. Transcription may exert its effects on *Xist* through counter-current polymerase collision or through topological constraints imposed on *Xist* by, for example, positive supercoils downstream of a moving *Tsix* polymerase complex. In Class III, Tsix RNA itself is the agent of repression. As Tsix and Xist RNAs can potentially form perfect duplexes, Tsix RNA could stoichiometrically titrate away Xist RNA, mask its functional domains, and/or facilitate its degradation. Indeed, Tsix RNA is present at 10–100-fold molar excess over Xist RNA [15], and its spliced forms overlap with *Xist*'s silencing domain [17]. However, because much of Tsix RNA seems to terminate before reaching *Xist* [15], we have questioned whether transcription of *Tsix*'s complementary region may be dispensable. In support of this idea, human TSIX RNA seems to terminate early [18, 19].

Truncating *Tsix* Reveals a Requirement for Antisense Transcription into *Xist*

To define specific molecular requirements, we generated new alleles of mouse *Tsix*. Because ES cells recapitulate XCI in culture, we targeted the female line, 16.7, which carries one X chromosome of *Mus musculus* (129) origin and one of *M. castaneus* (CAST) origin. To determine whether the 3′ half of *Tsix* is dispensable, we created *Tsix*^{Trap}, in which *Tsix* transcription is truncated at a BamHI site at the terminus of *Xist* (Figure 1B). Through a splice acceptor-IRES:Neo-polyA cassette (SA-Neo-pA), the SA directs splicing of proximal *Tsix* exons (E1a, 1b, 2, and 3) to the *Neo* selectable marker, whereas the *pA* truncates Tsix RNA distally. The 129 X was specifically targeted (Figures 1C–1E).

In parallel, we created *Tsix*^{hygro} as a control to exclude disruptive effects to the chromatin at the insertion site. In *Tsix*^{Hygro}, a CMV-driven *Hygro-thymidine kinase* fusion was targeted to the same BamHI site (Figures 1B and 1C). We also created the control, *Tsix*$^{Trap/TK}$, in which an *EF1*α-driven *thymidine kinase* ($P_{EF1\alpha}$-*TK*) is inserted into the *Tsix*^{Trap} cassette on the 129 X (Figures 1B–1D). This allele served as independent confirmation of any *Tsix*^{Trap} phenotype and as control for subsequent complementation (see below).

To determine whether *Tsix* transcripts were successfully truncated in *Tsix*^{Trap} and *Tsix*$^{Trap/TK}$ cells, we compared RNA levels at positions proximal and distal to the

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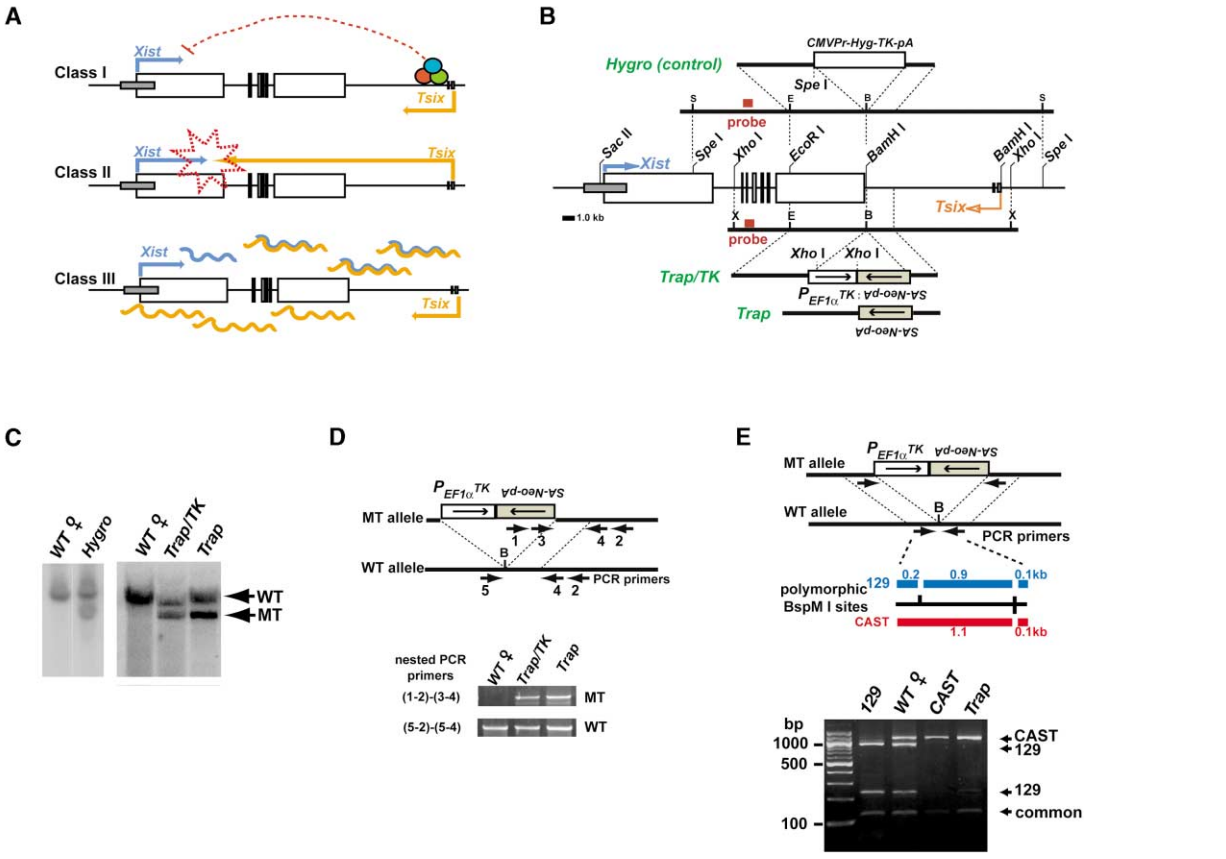


Figure 1. Current Models and Generation of *Tsix* Truncation Mutants

(A) Potential mechanisms of *Tsix* regulation. Class I are DNA- or chromatin factor-mediated; class II, transcription-mediated; and class III, RNA-mediated. *Xist* exons are shown in white, *Tsix* exons in gray.

(B) Targeting constructs. The probe for Southern analysis is in red.

(C) Genomic Southern analysis indicates that one X chromosome is targeted. For *Hygro*, genomic DNA is digested with SpeI and probed with the distal fragment indicated in (B). The wild-type (WT) band is 30 kb and the mutant (MT) band is 16 kb. For *Trap* and *Trap/TK*, DNA is digested with XhoI; the WT band is 23 kb and the MT band is 12 kb.

(D) PCR genotyping confirms correct targeting of the proximal end.

(E) Allele-specific PCR based on a BspMI polymorphism between 129 and *M. castaneus* (CAST) chromosomes revealed that the 129 allele was targeted in each mutant.

insertion site. Allele-specific RT-PCR of undifferentiated cells revealed that although antisense levels were comparable on the two X's at the most 5′ position, the levels were significantly reduced on the mutated X at positions distal to the truncation site (Figure 2A). The reduction in *Tsix* RNA was similar to that observed in $Tsix^{\Delta CpG}$, a promoter knockout of *Tsix* [6]. In contrast, wild-type female ES cells exhibited nearly equal transcript levels at all three positions along *Tsix*. Consistent with RT-PCR results, RNA fluorescence in situ hybridization (FISH) showed that antisense signals were detectable on the targeted chromosome with a 5′ but not 3′ *Tsix* probe (Figure 2B). In contrast, signals were detected at both positions on the untargeted homolog in mutants. The control cell line, $Tsix^{Hygro}$, showed no obvious changes in *Tsix* levels (data not shown), suggesting that interrupting the BamHI site had no effect on *Tsix* expression. These results demonstrated that the *Trap* and *Trap/TK* cassettes effectively truncated *Tsix* expression within the *Xist* gene body.

We next assayed the effects of *Tsix* truncation on *Xist* expression and inactivation of the representative X-linked gene, *Mecp2* (Figure 2C). To induce XCI, we differentiated ES cells by suspension culture into embryoid bodies (EB) for 4–11 days. Wild-type cells exhibited random XCI patterns, although there is a characteristic bias toward inactivating the 129 X because of the *Xce* modifier [20]. Intriguingly, in $Tsix^{Trap}$ and $Tsix^{Trap/TK}$ cells, skewing became extreme, with 97–98% of *Xist* transcripts originating from the mutated X. Consistent with *Xist* skewing, *Mecp2* expression heavily favored the normal X (Figure 2C). Importantly, the $Tsix^{Hygro}$ insertion had no apparent effect on *Xist* expression or XCI, which suggests that the skewing effect of $Tsix^{Trap}$ and $Tsix^{Trap/TK}$ was specifically the result of truncating the transcript rather than a disruption of critical sequences at the targeting site. These results indicated that truncating *Tsix* transcripts short of crossing *Xist* results in a loss of function in *Tsix*. Thus, antisense function specifically requires that *Tsix* synthesis traverse *Xist*.

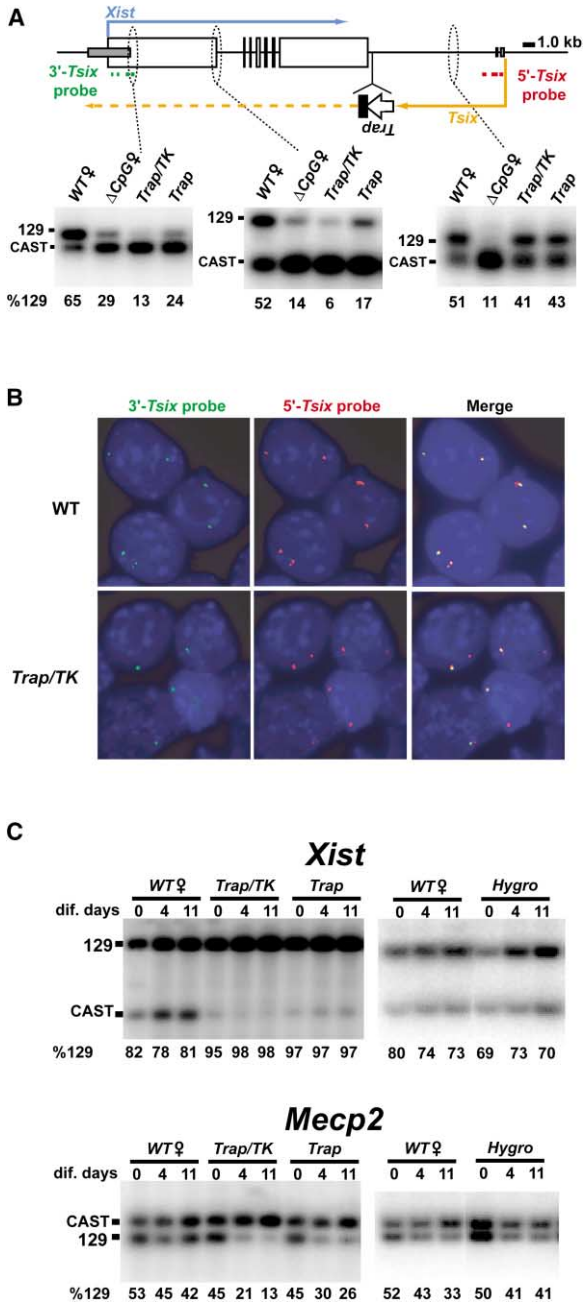


Figure 2. Characterization of Truncation Mutants

(A) Allele-specific RT-PCR of *Tsix* at various positions confirmed RNA truncation. The fraction of 129 transcripts is indicated below each panel. Allele-specific analysis at the 5′ position is based on a polymorphic MnlI site; the distal positions are based on ScrFI and SpeI.

(B) Two-color FISH analysis further confirmed *Tsix* truncation. Strand-specific *Tsix* riboprobes are as indicated in (A).

(C) Skewing of XCI revealed by allele-specific RT-PCR analysis of *Xist* and *Mecp2* expression on undifferentiated (d0) and differentiated (d4, d11) cultures. The %129 averages shown below each lane represent three independent experiments.

cis Complementation Restores Antisense RNA but not *Tsix* Function

The requirement for *Tsix* expression to cross *Xist* could depend on either antisense transcription per se or the RNA. To distinguish between them, we carried out RNA cis complementation, in which *Tsix* RNA is produced without concurrent transcription through *Xist*. We reasoned that if Tsix RNA were the acting agent, knocking in a *Tsix* minigene in *cis* might rescue the $Tsix^{Trap}$ truncation defect. We generated $Tsix^{cDNA}$, in which the 2.4 kb *Tsix* cDNA [21] is inserted downstream of the *SA-Neo-pA* cassette (Figure 3A) on the 129 chromosome (Figures 3B and 3C), ensuring *Tsix* expression in *cis* to the *Trap* mutation. To exclude the role of transcription, we specifically directed minigene synthesis in the same orientation as *Xist*. In principle then, we could restore *Tsix* RNA levels without concurrent antisense transcription through *Xist*.

To examine the expression profile of $Tsix^{cDNA}$, we carried out RT-PCR. Whereas $Tsix^{Trap}$ females expressed only half the amount of spliced Tsix RNA (presumably from the untargeted X), $Tsix^{cDNA}$ females exhibited wild-type levels (Figure 3D). Thus, $Tsix^{cDNA}$ effectively restored the quantity of processed Tsix RNA. We then asked whether the cDNA transcript localized to the *Xic*, *Tsix*'s recognized site of action. With a probe that specifically detected exon 4 of Tsix RNA, FISH revealed two tightly localized pinpoints at the *Xic* (Figure 3E)—a result that contrasted with the single pinpoint of $Tsix^{Trap}$ and $Tsix^{Trap/TK}$ (Figure 2B). RNAs made from the two X's of $Tsix^{cDNA}$ females were indistinguishable with respect to signal size, intensity, and localization. There was no apparent diffusion of Tsix RNA in the nucleus and cytoplasm, but FISH may not be sufficiently sensitive to detect low-level diffusion. These results demonstrated not only that the $Tsix^{cDNA}$ allele restored antisense RNA levels but also that at least a fraction of the cDNA transcript exhibited proper cis localization.

Because the minigene construct drove expression in the reverse orientation with respect to *Tsix*, a concern is that reverse orientation might disrupt events at the 5′ end of *Tsix* (12 kb away). To exclude this effect, we examined $Tsix^{Trap/TK}$ as a control. Notably, $Tsix^{Trap/TK}$ and $Tsix^{cDNA}$ have identical minigene architecture except for the substitution of *cDNA* for *TK*. We observed that the $Tsix^{Trap/TK}$ phenotype was similar to $Tsix^{Trap}$ (Figure 2), a construct that does not have any opposing minigene expression. Furthermore, we asked whether transcriptional activity from the major *Tsix* promoter itself had been affected (Figure 3F). Allele-specific RT-PCR analysis of endogenous *Tsix* expression showed essentially identical behavior of $Tsix^{Trap}$, $Tsix^{Trap/TK}$, and $Tsix^{cDNA}$ at all tested positions. FISH at the 5′ end of *Tsix* also showed signals of comparable intensity to that of the wild-type (Figure 3E). These findings argued that the cDNA knock-in did not affect endogenous expression.

To determine whether $Tsix^{cDNA}$ could complement the $Tsix^{Trap}$ defect, we differentiated the mutant cell lines and examined their XCI phenotype. RNA FISH analysis of $Tsix^{cDNA}$ female cells revealed the appearance of a single Xist RNA domain between days 3 and 8 in all cell lines; this domain was similar to that observed in wild-type and $Tsix^{Trap/TK}$ cells (Figure 4A), suggesting proper dosage compensation in $Tsix^{cDNA}$ cells. To address effects on XCI ratios, we carried out allele-specific RT-PCR analysis and found that XCI remained as highly skewed in $Tsix^{cDNA}$ as in the $Tsix^{Trap}$ mutant (Figures 4B

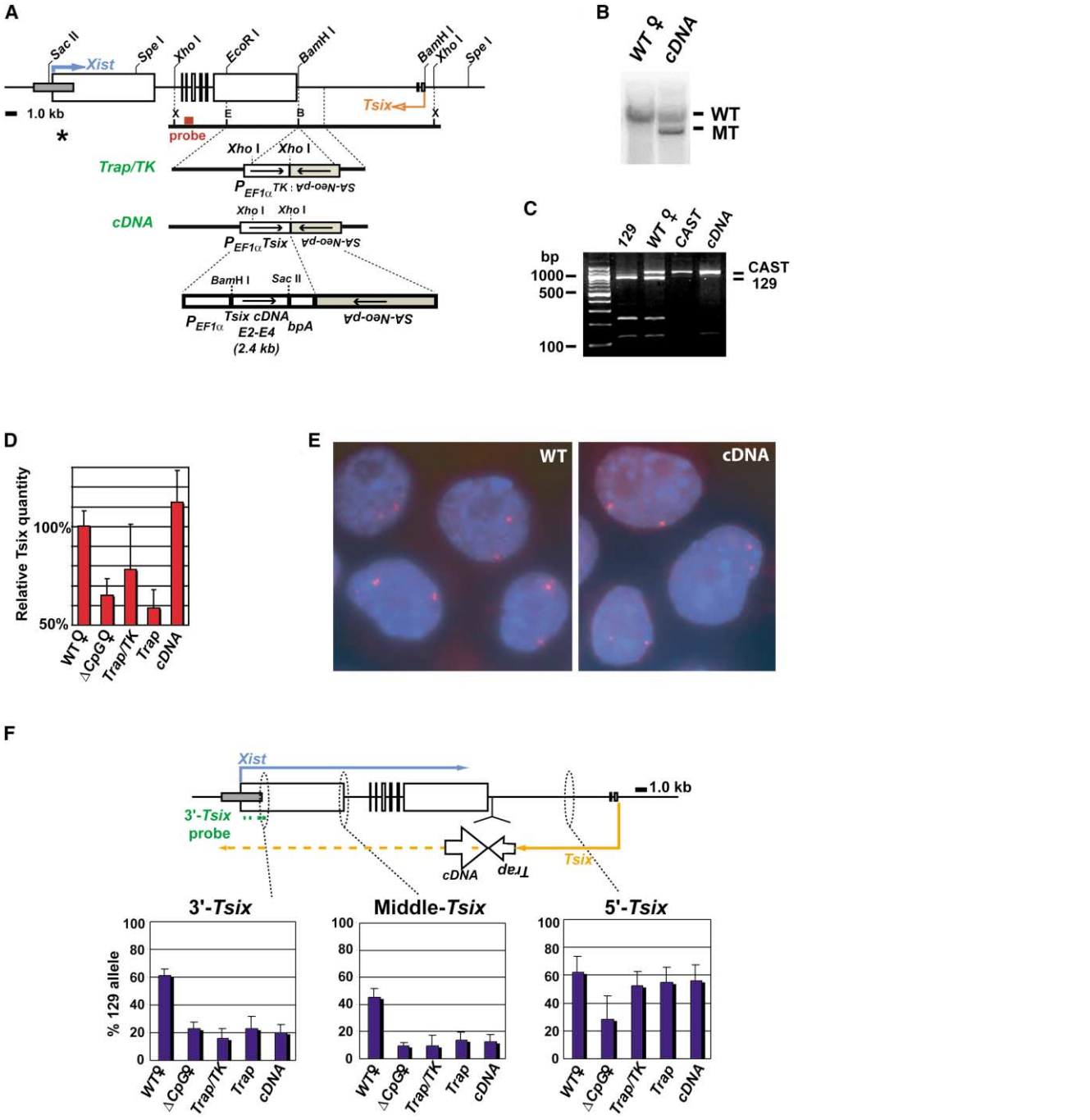


Figure 3. Generation and Characterization of *Tsix*^{cDNA}

(A) The *Tsix*^{cDNA} targeting construct.

(B) XhoI-digested genomic Southern blot analysis as described in Figure 1C allowed the integrity of the distal arm to be checked. The proximal homology was check by PCR as described in Figure 1D.

(C) Allele-specific PCR revealed targeting of the 129 allele as described in Figure 1E.

(D) Real-time, strand-specific RT-PCR showed that *Tsix*^{cDNA} restored antisense transcript quantity. The asterisk in panel A indicates the amplicon position.

(E) Using a *Tsix* exon 4-specific probe (red, panel F) to detect cDNA expression, RNA FISH analysis suggested that at least some of the minigene RNA is concentrated at the *Xic*.

(F) *Tsix*^{cDNA} did not affect endogenous *Tsix* at long range. Allele-specific RT-PCR at positions proximal and distal to the insertion site, as described in Figure 2A, are shown.

and 4C), suggesting that knocking in the antisense cDNA in *cis* cannot complement the loss-of-function phenotype of *Tsix*^{Trap}. Indeed, on day 11, Xist RNA originated almost exclusively from the 129 allele of *Tsix*^{cDNA} cells, with consequent bias of *Mecp2* expression to favor the *castaneus* allele. These findings demonstrated that re-

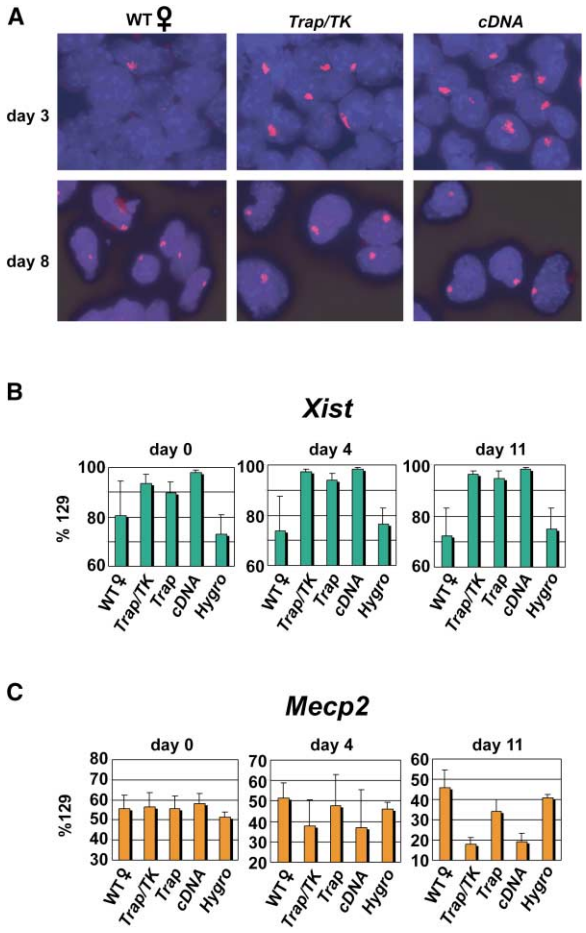


Figure 4. *Tsix*^{cDNA} Failed to Rescue Antisense Function

(A) RNA FISH demonstrated upregulation of *Xist* in differentiated cultures on days 3 and 8. Xist RNA is in red.

(B and C) Allele-specific RT-PCR analysis of *Xist* and *Mecp2* expression in wild-type and mutants cells on days 0, 4, and 11 of differentiation. The 129 fraction (average and standard deviations) is plotted on the histograms and is derived from three independent experiments.

storing the quantity of the processed antisense RNA alone cannot rescue the function of *Tsix*.

We conclude that the spliced antisense transcript is not sufficient for *Tsix* function, nor is transcription through the noncomplementary region of *Tsix*. Without restoration of transcription through *Xist*, restoring *Tsix* RNA levels could not rescue *Tsix* function. These findings suggest that *Tsix* action does not merely derive from processed antisense RNAs (class III) but requires concurrent antiparallel transcription through *Xist* (class II). It is striking that Tsix RNA is spliced to remove almost all complementarity to *Xist* except for a 1.9 kb region that overlaps with *Xist*'s silencing domain [17], a point which makes an RNA titration mechanism particularly attractive. The available evidence leads us to favor a combination of class II and class III mechanisms in which *Xist* repression requires the antisense RNA but the RNA must be synthesized concurrently off the complementary strand (Figure 5C). We suggest that cotranscription provides a higher local RNA concentration than is achievable by overexpressing *Tsix* in *cis* at a downstream position—perhaps the cDNA's downstream placement constrained the RNA's diffusion and topologically precluded interaction with Xist RNA. Other possible reasons for failed cDNA complementation include insertion of an inert splice variant (*Tsix* has multiple splice variants [21, 15]) and the need for the full-length isoform.

X Chromosome Choice Depends on *Tsix* Transcription

Given a role for *Tsix* in X inactivation choice, we lastly tested the new *Tsix* mutations for possible effects on choice. From the allele-specific analysis of *Xist* and *Mecp2* expression (Figures 2C, 4B, and 4C), we did indeed observe skewed XCI ratios in *Tsix*^{Trap}, *Tsix*$^{Trap/TK}$, and *Tsix*^{cDNA} heterozygotes. However, a priori, any observed nonrandom pattern of XCI could result from either a primary effect that truly precludes the selection of one X, or a secondary (perceived) effect due to lethality of choosing the "wrong" X for silencing. In the latter case, both X's are competent to be chosen, but the demise of cells that have chosen the incorrect X results in a perceived skewing of the XCI ratio.

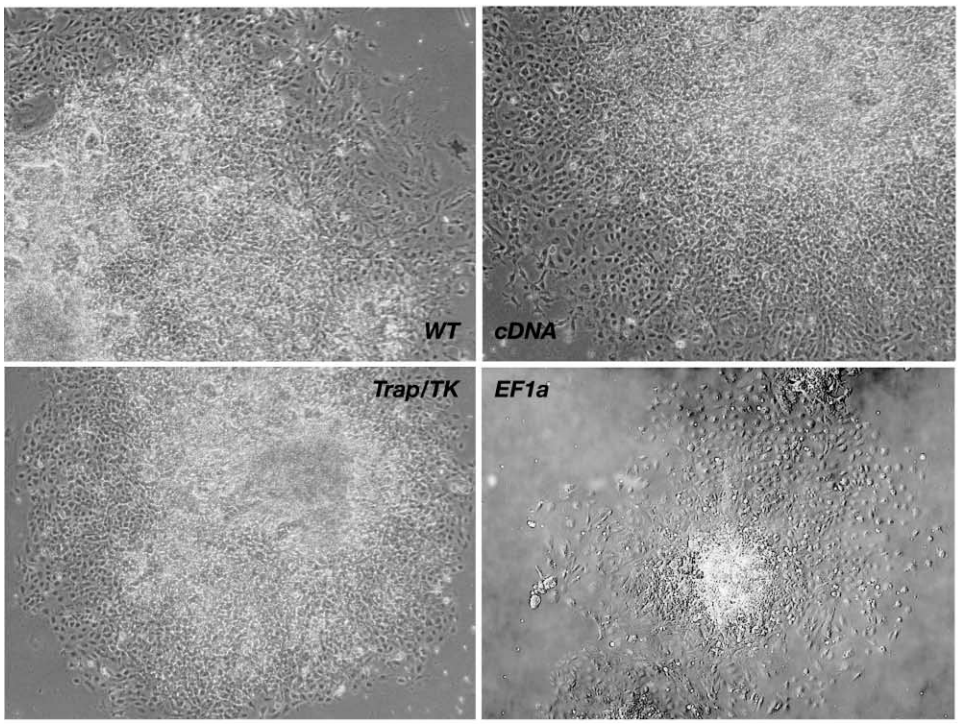
To determine the cause of skewing, we carried out cell death assays during the time window (days 0–7 of differentiation) when XCI takes place in ES cultures. At a gross level, wild-type and mutant EBs looked indistinguishable with respect to the quantity, cell types, and morphology of EB outgrowths (Figure 5A and data not shown). Trypan blue staining of all cells (both EB and extruded cells) in suspension culture showed that the rates of cell death were comparable to those of the wild-type (Figure 5B). These results suggested that the mutations in *Tsix*^{Trap}, *Tsix*$^{Trap/TK}$, and *Tsix*^{cDNA} prevented the mutated X from being chosen as the Xa, thereby supporting a primary mechanism of allelic skewing. If the mutated X could have been chosen as the Xa (and the normal X as Xi), cells that chose to do so would have died because of the silencing of both X's. In contrast, *Tsix*$^{EF1\alpha}$ heterozygotes—a mutant previously shown to exhibit secondary nonrandom XCI [13]—showed increased cell death and stunted EB growth (Figures 5A and 5B; note the relatively "anemic" quality of the *Tsix*^{EF1a} EB). We conclude that *Tsix* transcription across the *Xist* locus is required for random choice and, in its absence, restoration of processed Tsix RNA alone is not enough to restore the choice decision.

A Feedback Model for Epigenetic Choice

An earlier study demonstrated that *Tsix* hypertranscription is not sufficient to influence choice, but it left open the possibility that *Tsix* transcription may be necessary [13]. The present mutants revealed that this is indeed the case, in that disrupting *Tsix* transcription before crossing *Xist* abolished selection of the linked X as the Xa. Restoring Tsix RNA levels did not rescue the ability to choose that X. Therefore, *Tsix* transcription is necessary, but not sufficient, for choice. Chromatin-associated elements that have been proposed to play a role in choice include the repeat, *DXPas34* [22], and binding sites for the CTCF chromatin insulator [23] at the 5′ end of *Tsix* [16]. Thus, it seems that a class I mechanism must be invoked in epigenetic choice in addition to class II and class III mechanisms.

How do we explain the complex interplay of *Tsix* chro-

A



B

% cell viability

	day 0	day 2	day 4	day 6
WT	>99%	95%	89%	89%
Tsix-Trap/TK	>99%	94%	91%	88%
Tsix-cDNA	>99%	89%	82%	84%
Tsix-EF1α	>99%	91%	59%	44%

C

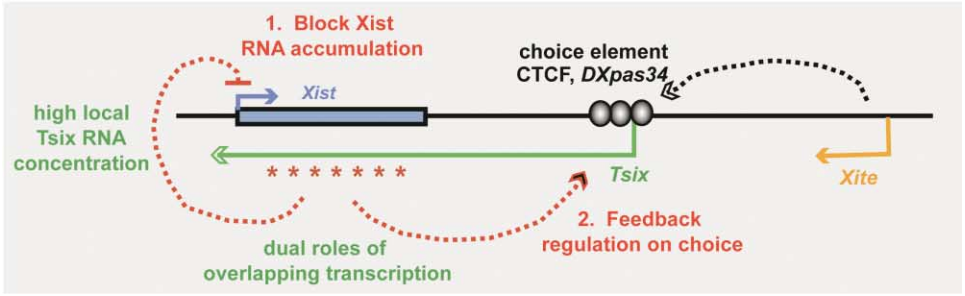


Figure 5. A Primary Effect on X Chromosome Choice Suggests a Feedback Mechanism of Regulation

(A) Healthy differentiation of heterozygous *Tsix*$^{Trap/TK}$ and *Tsix*^{cDNA} cultures into day 7 embryoid bodies. In contrast, heterozygous *Tsix*$^{EF1\alpha}$ cultures were stunted in growth and exhibited greater cell death.

(B) Trypan blue assays to quantitate cell death on days 0–6 of differentiation. Multiple differentiation experiments were carried out with 10^5–10^6 cells on day 0 for the wild-type, *Tsix*$^{Trap/TK}$, and *Tsix*^{cDNA} mutants. A few hundred cells were counted in each trypan blue assay. Because differentiation conditions vary between experiments, one representative experiment is shown.

(C) An integrated model of feedback regulation in which both functions of *Tsix*—choice and *Xist* repression—require overlapping transcription through the *Xist* locus. Asterisks mark the "business end" of Tsix RNA. See text for details.

matin and transcription/RNA in the mechanism of choice? We suggest that it reflects the inherent intricacy of the choice decision, during which two identical X's must adopt opposite epigenetic fates. In principle, this decision must have a "trans" component for communication between the homologs; this component would ensure mutual exclusion of Xa and Xi fates [24]. Furthermore, if multiple elements regulate X inactivation choice,

as appears to be the case (*Xce* [20, 25], *Xite* [26], *Tsix* [6], and *Xist* [27, 28]), there must be coordination in "*cis*" among all elements.

We propose a feedback system that would ensure successful stepwise selection of one and only one Xa (Figure 5C). Hypothetically, the binding of specific transcription factors (e.g., CTCF) and the action of *Xite*, a positive regulator of *Tsix* [26], would lead to activation and persistent expression of *Tsix* in *cis*. However, factor binding alone would not be sufficient to lock in the Xa fate. Successful traversal of *Tsix* transcription through *Xist* would be required to ensure the Xa fate. If transcription were prematurely terminated, the mechanism could bypass this chromosome in favor of its homolog, where it would make a second attempt at securing the Xa. Such a mechanism is conceptually similar to feedback regulation in immunoglobulin [29] and olfactory receptor choice [30], in which the successful display of one gene product precludes further choice. The mechanism may explain published accounts indicating that *Xist* mutations affect choice [27, 28]. Certainly, the effectiveness of such a feedback system would be enhanced by including checkpoints throughout, from *Xite* to *Tsix* to *Xist*; this would avert potentially lethal decisions arising from nonproductive Xa selection. Because the choice of Xi is mitotically stable in somatic cells, the reversibility must be specific to the early embryo. Given the critical nature of choice, mammals would be well served by a mechanism that is self-patrolling and transiently reversible.

Experimental Procedures

Targeting Constructs

The SA-IRES-neo-pA cassette was derived from pGT1.8IresBgeo [31] through the removal of β-galactosidase. The 5′ homology arm contains a 6.7 kb EcoRI-BamHI fragment from exon 7 of *Xist*. The 3′ arm was PCR amplified from 129 sequence by *Pfu* with primers 5′-GTA AAG CAC AGA GGA ACT AG*T TAA CCT GAG-3′ (G* is an altered base that generates a SpeI site for cloning; it is lost after recombination) and 5′-TCG GAT CCC AAG GAA ACC AAG TTA C-3′. The product was digested with BamHI and SpeI, subcloned into pBluescript, and completely sequenced. The *EF-1α* promoter has been described [13]. The *bpA* was obtained from pPGKneobpA [32]. The *Tsix* cDNA minigene included exons 2–4 and was obtained through the ligation of the 1.9 kb BamHI-SacII fragment of pB1-S plasmid to the 0.5 kb BamHI fragment of pE14 [15] into pBluescript vector.

Gene Targeting in ES Cells

16.7 ES cells [6] were electroporated with targeting vectors, selected in 300 μg/ml G418 at 24 hr, and picked on day 9. For genotyping, genomic DNA was digested with XhoI (for $Tsix^{Trap}$, $Tsix^{Trap/TK}$, and $Tsix^{cDNA}$) or with SpeI (for $Tsix^{Hygro}$) and probed with a 0.9 kb HindIII-BamHI *Xist* fragment spanning exons 3 and 4 (bp 3356–4219 of Genbank U41394) by Southern analysis (integrity of distal arm). To assess the integrity of the proximal arm, we performed nested PCR with *AmpliTaq Gold* DNA polymerase (Applied Biosystems) with primers (1) 5′-TCC TAC AAC ACA CAC TCC AAC CTC-3′, (2) 5′-TCT TAT CCA CTC CAC CTT CTC TCC-3′, (3) 5′-TCG GTT CCT CTT CCC ATG AAT TCC-3′, (4) 5′-TTT GGT TCG AGT AAA GCA CAG AGG-3′, and (5) 5′-GTT TGG GTG TTA TAC CCG TGT AGG-3′, with parameters 94°C/7 min (one cycle); 94°C/30 s, 66°C/30 s, and 72°C/3 min (45 cycles); and 72°C/10 min (one cycle). To determine which chromosome was targeted, we performed allele-specific PCR with primers 5 and 5′-GTG TCC CAA TCC TTT AGT TGC CAG-3′ with the same program except for a 1 min elongation time and then restriction digestion with BspMI enzyme.

Allele-Specific RT-PCR

RNA was prepared with Trizol (Invitrogen), and the RT reaction was carried out with Superscript II (Invitrogen) by random hexamer priming. Protocols for *Xist*, *Mecp2*, 5′-*Tsix*, and middle-*Tsix* have been described [13]. For 3′-*Tsix*, cDNA was prepared with a strand-specific primer (5′-GGT GCT CAG ACA ACA ATG-3′), and RT-PCR spanned a polymorphic SpeI site with primers 5′-GGA GAG CGC ATG CTT GCA ATT CTA-3′ and 5′-TAG AGA ACC GCT TGA GAT CAG TGT-3′. A nested probe, 5′-GGT TTC AAT GAT TTA CAT CGA CCA AGA ACC CGC AGC CTC G-3′, was used for detection by Southern analysis, and relative probe intensities were quantitated by phosphorimaging. For real-time PCR quantitation of Tsix RNA (Figure 4A), RT was carried out with a gene-specific primer, 5′-GTG TGA GTG AAC CTA TGG-3′, and the products were amplified with amplicon 8 primers as previously described [15].

RNA FISH

FISH was performed as described [6]. 5′-*Tsix* probes were prepared from pNS2, pE3SD, and pC7PM [15]. 3′-*Tsix* probes were from pE1BE2 (BamHI-EcoRI fragment in *Xist* exon 1, bp1877-2379 of Genbank L04961), pE1BMB (BspMI-BamHI fragment, bp1468-1877 of L04961), p@840 (bp840-1181 of L04961), and p@72 (bp72-390 of L04961).

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